POSITIVE INCREASE OF REDOX POTENTIAL OF THE EXTRAMITOCHONDRIAL NADP(H) SYSTEM BY MIXED FUNCTION OXIDATIONS IN HEMOGLOBIN-FREE PERFUSED RAT LIVER

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1. Introduction

The use of indicator metabolite systems and of suface fluorimetry for evaluation of potentials of NADH/NAD$^+$ in specific subcellular compartments of liver has recently been summarized by Bücher [1].

With respect to the potential of NADPH/NADP$^+$, Krebs and Veech [2] have extended the indicator metabolite approach to several NADP(H)-dependent enzyme systems in the extramitochondrial space. A contribution of NADPH to the fluorescence signal was recently identified in perfused liver during high rates of mixed function oxidation [3]; with hexobarbital as substrate, there was a substantial decrease of fluorescence intensity. Concomitantly, the tissue level of NADPH decreased, while the potential of the cytosolic free NADH system remained unaltered.

On the basis of the findings mentioned above, in the present paper a more detailed analysis of the changes in indicator metabolite couples and in tissue levels of the NADPH/NADP$^+$ and NADH/NAD$^+$ systems during mixed function oxidations in livers of phenobarbital-treated rats has been made with the following results: (a) as indicated by the malic enzyme system, a potential change to the positive occurs in the extramitochondrial free NADPH/NADP$^+$ system; (b) as indicated by the lactate dehydrogenase system, no potential change occurs in the cytosolic free NADH/NAD$^+$ system; (c) coordination of these systems via the common reactant, pyruvate, is observed (see [2]); (d) the potential of the cytochrome *P*-450 system, tentatively calculated from degrees of reduction measured by absorbance photometry of perfused liver [3–5], shifts substantially to the negative.

2. Experimental

Livers from male Wistar rats, 120–150 g weight, fed on stock diet, were perfused with bicarbonate-buffered salt solution, pH 7.4, 37°, containing 7 g of dextran (M.W. 40,000; kindly supplied by Knoll Co., Ludwigshafen) per 100 ml, as described [3]. The animals received sodium phenobarbital at a concentration of 1 mg/ml with the drinking water for five days prior to perfusion, a treatment causing several-fold proliferation of the hepatic microsomal mixed function oxidase activity [6].

Reduced pyridine nucleotide surface fluorescence was measured with the Ultropak technique [7], using light of 366 nm from a mercury arc lamp for excitation. Emitted light of wavelengths > 420 nm was detected by an RCA 1P21 photomultiplier. Electronics were from Netheler and Hinz, Hamburg.

Tissue level analyses were performed from freeze-stopped tissue. Liver samples (500 mg) were taken from the lower left liver lobe where surface fluorescence was monitored. NADPH and NADH were extracted with 0.5 N alcoholic KOH for 5 min at 90° and were converted to the more stable oxidized forms after neutralization, according to Klingenberg [8]. NADP$^+$ and NAD$^+$ as well as L-malate, L-lactate and pyruvate were extracted with 0.6 N perchloric acid and assayed fluorimetrically using an Eppendorf fluorimeter (cf. [9]). Data given are uncorrected for recoveries which ranged between 90 and 95%. Tissue levels are denoted by { }, concentrations by [], according to [10].

Oxygen uptake was followed with Ag-Pt (20 μm)-

microelectrodes inserted into the perfusion circuit before and after the liver. Normoxic conditions prevailed also during high rates of mixed function oxidation [3].

3. Results

3.1. *Surface fluorescence and oxygen uptake*

The rapid decrease of reduced pyridine nucleotide surface fluorescence following the addition of 40 μmoles of sodium hexobarbital to the perfusion fluid (100 ml) of the liver of a phenobarbital-treated rat is shown in fig. 1 (see also [3]). Concomitantly, oxygen uptake increases by about 50% over the initial rate of 1.8 μmoles O_2 per min per g fresh weight of liver. With the assumption that other O_2 consuming systems operate at a constant rate and that the reaction is NADPH-specific, this would correspond to a rate of about 0.9 μmole NADPH oxidized per min per g fresh weight of liver.

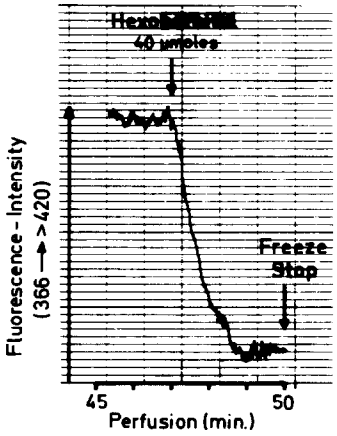

Fig. 1. Decrease of intensity of reduced pyridine nucleotide surface fluorescence following the addition of sodium hexobarbital to hemoglobin-free perfused liver from a phenobarbital-treated rat.

3.2. *NADP(H) system*

When a new steady state had established after the addition of hexobarbital, tissue samples were taken by freeze stop at 50 min (see fig. 1). The data of tissue level analyses are given in table 1 and compared to controls. The {NADPH}/{$NADP^+$} ratio is lowered from 4.0 in the controls to 2.2 in the presence of hexobarbital [3]. In contrast, the {NADH}/{NAD^+} ratio remains unchanged at 0.1. {L-Malate}, linked to the NADP(H) system by malic enzyme, decreases to about half of the level in the controls, while the level of the product of the malic reaction, pyruvate, remains constant. With the assumption of equilibrium of the malic enzyme system (tissue level of activity: 1.27 U per g fresh weight of liver of untreated fed rats [2]) with the extramitochondrial NADP(H) system, and of constant [CO_2] of 1.2 mM (this is the [CO_2] of the incoming perfusate), potentials may be calculated from tissue levels using the Nernst equation. Although incorrect thermodynamically, tissue levels

Table 1
Effect of sodium hexobarbital on the NADP(H) and NAD(H) systems in perfused livers of phenobarbital-treated rats.

Tissue levels at 50 min of perfusion	{NADPH}	{$NADP^+$}	{NADH}	{NAD^+}	{L-Malate}	{Pyruvate}	{L-Lactate}
	(nmoles per g dry weight of liver)						
Controls	883 ± 26	218 ± 6	183 ± 7	1,864 ± 56	545 ± 71	552 ± 38	8,230 ± 849
Sodium hexobarbital (40 μmoles) added at 47 min	739 ± 28	329 ± 15	169 ± 11	1,704 ± 74	245 ± 33	581 ± 34	8,463 ± 816
n	10	7	10	7	5	3	3

Tissue levels of the pyridine nucleotide couples and two dependent indicator metabolite couples representative of the potential of extramitochondrial free NADP(H) and NAD(H) are given. The metabolites are those of malic enzyme and lactate dehydrogenase, respectively. Data are given as nmoles per g dry weight of liver ± S.E.M. Number of experiments indicated below. Tissue samples had a water content of 4.69 ± 0.10 ml H_2O per g dry weight (n = 74).

may be used in an approximation since ratios of levels are employed [10, 11]. For the malic enzyme system, a midpotential of −382 mV is taken, calculated from the equilibrium constant of 3.44×10^{-2} [2] and a midpotential of the free pyridine nucleotide system of −337 mV (38°, $I = 0.25$, pH 7.0; see [1]). A positive increase of the potential of this NADP(H)-dependent indicator metabolite system by 12 mV (−382 → −370 mV) is calculated (table 2).

Considering that 1/3 of total {NADPH + $NADP^+$} is extramitochondrial [12, 13], {NADPH}/{$NADP^+$} would need to decrease only from 4.0 to 2.9 to account for the potential change. Since {NADPH}/{$NADP^+$} is observed to decrease to 2.2, there may be an indicator error in the extramitochondrial malic enzyme system. Another possibility is that the mitochondrial NADP(H) system also participates in the redox changes.

3.3. *NAD(H) system*

As stated above, the {NADH}/{NAD^+} ratio remains unchanged at 0.1 when the rate of mixed function oxidation is increased. Similarly, the cytosolic free NAD(H) system, assessed by ratios of [lactate]/[pyruvate] in the perfusate, does not undergo potential changes [3, 14, 15]. This is also shown in table 2

Table 2

Redox potentials of extramitochondrial NAD(H)- and NADP(H)-dependent indicator metabolite systems and of the cytochrome *P*-450 system as a function of the rate of mixed function oxidations in perfused livers of phenobarbital-treated rats. Midpotentials and the assumptions made are discussed in the text. Data from table 1 from refs. [3–5].

	Redox potentials in mV		
	$\frac{\{\text{L-Lactate}\}}{\{\text{Pyruvate}\}}$	$\frac{\{\text{L-Malate}\}}{\{\text{Pyruvate}\}}$	Cytochrome *P*-450
	$[CO_2] = 1.2$ mM		
Controls	−251	−382	−337
Hexobarbital (40 μmoles)	−251	−370	−386
Aminopyrine (20 μmoles)	–	–	−352

where potentials are calculated from ratios {lactate}/{pyruvate} of table 1, using a midpotential of −215 mV [1, 2] for this indicator metabolite system.

3.4. *Coordination of extramitochondrial NADP(H) and NAD(H) systems*

The absence of a potential change in the cytosolic free NAD(H) system concomitant with the positivation in the free NADP(H) system is not unexpected. While Krebs and Veech [2] found that the potential difference between these two systems was about −150 mV [11] in different nutritional states

$$\frac{[NAD^+]/[NADH]}{[NADP^+]/[NADPH]} \approx 10^5 \quad ,$$

they pointed out that the potential difference is not necessarily fixed at a given value but a function of the substrate levels. In the present case, {pyruvate}, the common linking reactant between the malic enzyme and lactate dehydrogenase systems, remains constant. Therefore, free [NADPH]/[$NADP^+$] may be also calculated from the {malate}/{lactate} ratios and proper constants. The suggestion by Thurman and Scholz [14, 15] that the extramitochondrial NADH and NADPH pools are not in rapid equilibrium, based on the observation of constant [lactate]/[pyruvate] ratios in the perfusate when aminopyrine was metabolized, therefore appears to require reevaluation.

3.5. *NADP(H) system and cytochrome P-450*

Waterman and Mason [16] have determined the midpotential of cytochrome *P*-450 to be −410 mV from measurements on isolated rat liver microsomes in the absence of substrate. As mentioned above, potentials may now be calculated using degrees of reduction obtained from absorbance photometry of perfused liver. CO titrations in the absence of external substrate yielded a degree of reduction of 0.06 [4], while in the presence of aminopyrine and hexobarbital as substrates, it increased to 0.10 [5] and 0.29 [3], respectively, as derived from steady state levels of the reduced cytochrome *P*-450–CO complex. Corresponding potentials, calculated for a one-electron process, are given in table 2. These preliminary data indicate that the potential of cytochrome *P*-450 should be at least 16 mV more positive when hexobarbital is present and even more so if there is an indicator error in

the malic enzyme system (see above). The possibility exists that the midpotential of *P*-450 is more positive than −410 mV in the intact organ, and also that the midpotential shifts to the positive when substrate is bound.

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